Synbiotic potential of colored rice flour as wall material and encapsulated with *Lactobacillus plantarum* TISTR 1465:Its impacts on gut bacterial population and metabolic activities

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Abstract

Black waxy and red jasmine rice flour (6.5 and 18.7% amylose content, respectively) were modified using pullulanase followed by heat-moisture treatment (hydrolyzed-HMT) to produce potential synbiotic microencapsulation with *Lactobacillus plantarum* TISTR 1465. Hydrolyzed-HMT of colored rice flours showed restricted pasting properties, lower breakdown and higher thermal properties than native flour ($P < 0.05$). Hydrolysis treatment was able to promote a low molecular weight starch that easily formed a crystalline structure after HMT. As a consequence, a significant increase in slowly digestible starch (23.65% to 36.96%) and resistant starch (11.41% to 14.36%) and a decrease in rapidly digestible starch (47.70% to 40.30%) were more noticeable in waxy flour than native flour. Microcapsules made from black waxy rice flour hydrolyzed for 36 h followed by HMT (hydrolyzed 36h-HMT) obtained the highest survival rate of *L. plantarum* (89.56%), even after longer storage time (90 days, 4ºC). In the stage of simulated gastric fluid, the survival rate of *L. plantarum* in hydrolyzed 36h-HMT microcapsules was much higher (88.02%; 8.07 Log CFU/g) than gum arabic (75.38%; 6.12 Log CFU/g) and no carrier (36.86%; 3.34 Log CFU/g). At the end of simulated intestinal fluid, hydrolyzed 36h-HMT showed much higher survival (81.56%; 7.48 Log CFU/g) than gum arabic (58.1%; 4.72 Log CFU/g) and no carrier (0%). Under scanning electron microscopy, starch granules of the hydrolyzed 36h-HMT were seen as polyhedral shapes in the spherical aggregates that carried the microorganisms and reduced their injury and mortality. Short-chain fatty acids of the hydrolyzed 36h-HMT were much higher than positive control at every fermentation time ($P < 0.01$). The fluorescence *in situ* hybridization data showed that the prebiotic property of hydrolyzed 36h-HMT can better aid the beneficial probiotic *Lactobacillus spp.* growth after 24 h fermentation than the negative control (from Log 8.40±0.50 to 7.03±0.20, $P < 0.05$) and commercial prebiotic Orafti®Synergy1 (Log 8.40±0.50 to 7.47±0.08, $P < 0.01$). Microencapsulation of hydrolyzed black waxy rice flour followed by HMT is proposed as a potential synbiotic ingredient to apply in functional foods, further studies of these novel formulations are needed to determine in vivo cell delivery performance and efficacy.

Keywords: black waxy rice; pullulanase; heat mositure treatment; synbiotic; fluorescence *in situ* hybridization

Chemical compounds: 1,1-diphenyl-2-picrylhydrazyl (PubChem CID:74358); 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (PubChem CID: 16240279); Trolox (PubChem CID: 40634)

Abbreviations

Hydrolyzed-HMT	:	Hydrolyzed heat-moisture-treated rice flour
FISH	:	Fluorescence in situ hybridization
SCFAs	:	Short-chain fatty acids
SDS	:	Slowly digestible starch
RS	:	Resistant starch

1. Introduction

Slowly digestible starch (SDS) and resistant starch (RS) are defined as the slow- to non-digestible portion of starch that cannot be digested after ingestion for 20-120 min and longer than 120 min, respectively. Indigestible carbohydrates, which can pass through the upper part of the gastrointestinal tract into the large intestine, are subsequently fermented by gut microbes (Zhang & Hamaker, 2009; Wang et al., 2002; Casterline et al., 1997). These prebiotic carbohydrates should be able to provide an absorption site for probiotic adherence and, at the same time, a carbon source for probiotic bacteria. These results in favorable metabolites such as short-chain fatty acids (SCFAs) in the human colon (Brouns et al., 2002; Crittenden al., 2001; Johnson & Gee, 1996). SDS and RS are known to aid human health benefits, such as stable glucose metabolism, reduced risk of diabetes, obesity, cardiovascular disease and colonic cancer (He, Liu, & Zhang, 2008; Lehmann & Robin, 2007). RS content of native colored rice from indigenous Thai rice varieties is about 5.33-5.69% (Pongjanta, Chomsri, & Meechoui, 2016). Factors affecting SDS and RS formation are crystallinity, chain length distribution, amylose and amylopectin ratios, and retrogradation (Shi & Gao, 2011; Miao, Jiang, & Zhang, 2009). SDS and RS can be produced by physical, chemical and enzymatic modifications. Physical modification is considered safe for human consumption.

The dual modification proposed in this study was a combination of two safe processes: heat-moisture treatment (HMT) and enzymatic modification. Significant increase in SDS (40.8 %) was found after HMT of native brown rice (Chung et al., 2012). Enzymes such as α amylase, β amylase, amylo-sucrase, pullulanase and iso-amylase were often used to cleave the outer chain of amylopectin, hence increasing starch chain mobility which led to a highly crystalline

structure. Shorter amylose chains were usually required, accounting for its ability to be readily re-associated into a more orderly crystalline structure (Shi & Gao, 2011). For SDS and RS production, amylo-sucrase was reported to be able to hydrolyze starch chains in waxy/normal rice and potato starch into DP 25-36 that aided crystalline perfection (Shin et al., 2010). Pullulanase treatment followed by repeated retrogradation was reported to increase SDS and RS content (25.4% and 50.1% respectively) in waxy maize starch (Miao, Jiang, & Zhang, 2009). The most SDS was reported to be produced by debranching waxy starch with pullulanase for 4 h and subsequent storage at 1°C. Waxy starch is more suitable to make SDS (Guraya, James, & Champagne, 2001). Starch that was hydrolyzed and allowed to crystallize showed higher RS than those only debranching (Cai & Shi, 2010). The co-process of hydrolysis followed by HMT was reported to increase the ratio of linear glucan α-D-(1, 4), which supported crystalline structure and resulted in a more enzymatic-resistant starch (Mutungi et al., 2010; Lin et al., 2009).

Various polysaccharides that have been used as encapsulating materials include gum arabic, inulin, oligosaccharides, maltodextrin and resistant starch (Perdana et al., 2014; Soukoulis et al., 2014a; Fritzen-Freire et al, 2013; Fahimdanesh et al., 2012; Desmond et al., 2002). These polysaccharides have different degrees of prebiotic effects, depending on its structure and composition that favor probiotic growth. The carrier matrix of gum arabic and sodium caseinate in low-melting-point fat microparticles was reported to enhance probiotic survival after spray drying, storage and *in vitro* digestion (from 1.20 Log CFU/g to 2.55 Log CFU/g) (Liu et al., 2016). Prebiotic edible films made from native rice and corn starch, mixed with gelatine, sodium caseinate and soy protein concentrate were used to encapsulate *L. rhamnosus* GG in a bread coating. The viability of *L. rhamnosus* GG is increased by 3 to 7-fold under simulated gastro-

intestinal conditions (Soukoulis et al., 2014b; Soukoulis et al., 2016). Glucose-oligosaccharides and polydextrose were also reported to enhance *L. rhamnosus* GG viability in prebiotic edible films during air drying. Inulin was the most effective material to maintain sub-lethal amounts of *L. rhamnosus* GG during storage (Soukoulis et al., 2014a).

The nutritious colored rice of interest in this study accounted for high antioxidant activity. The anthocyanin content in black/purple, red and wild rice were 3276.0, 93.5 and 27.2 µg/g, respectively (Abdel-Aal et al., 2006). The synergistic effect of malvidin-3-glucoside mixed with other anthocyanins was reported to improve the growth of the good bacteria (Hidalgo et al., 2012). The colored rice flour was modified by the dual process of using HMT and enzymatic modification to obtain appropriate degrees of slow to indigestible prebiotic starch. Highly indigestible starch aids probiotic survival but lowers its utilization as a carbon source. On the contrary, rapidly digestible starch lowers probiotic protection in the gut system but serves as a good carbon source. Therefore, this study aimed to evaluate this trade-off effect using *in vitro* human faecal batch fermentation that closely mimics the real human gut system.

2. Materials and Methods

2.1. Preparation of hydrolyzed-HMT rice flour

Paddy rice of the black waxy “kam leum pua variety” (Phrae Rice Research Center in Phrae, Thailand) and the “red jasmine variety” (Khonkaen Rice Research Center in Khonkaen, Thailand) were dehulled to obtain brown rice grains. The brown rice was steeped in water for 3 h and then wet-milled using a double-disk stone mill to produce a 10% (w/v) rice flour slurry. The flour slurry was adjusted to pH 4.5 with a 0.1 M sodium acetate buffer. The enzyme pullulanase (OPTIMAX® L-1000, 1000 ASPU/g, Siam Victory Chemicals, Thailand) (0.2 g) was added into

the flour suspension (110 g) to obtain a concentration of 20 ASPU/g of flour (dry basis). The solution was incubated at 55°C for 8, 24 and 36 h in a shaking water bath. The solution was centrifuged (3000 *g*) for 10 min, the precipitate was washed twice with distilled water and collected by centrifugation (Miao, Jiang, & Zhang, 2009). The precipitate was oven dried at 40°C until the target HMT moisture content (25%) was obtained. The rice flour sample was then put in a sealed screw-cap container and equilibrated at room temperature for 24 h. The equilibrated containers were then placed in a hot air oven (100°C) for 1 h. After that, the treated flour was taken out and dried in a hot air oven (40 °C) until a 12% moisture content was obtained. The obtained sample or "hydrolyzed-HMT" rice flour was milled (ultra-centrifugal mill type ZM1, Retsch GmbH, Germany) and sieved to a particle size of 100 mesh, put in sealed plastic bag and kept at 4°C.

2.2. Physico-chemical properties of hydrolyzed-HMT rice flour

2.2.1. Pasting properties

The pasting property of the hydrolyzed-HMT rice flour was determined by the Rapid Visco Analyzer (model RVA3D; Newport Scientific, NSW, Australia). The hydrolyzed-HMT rice flour (3.00±0.01 g) was mixed with distilled water (25 mL) in a metal RVA canister (AACC Method 61-02, 2000). The sample suspension was heated in the RVA using the heating profile for rice flour. The sample was heated from 50°C to 95°C at rate 12°C/min and held at 95°C for 2.5 min, cooled down to 50°C at a similar rate and held at 50°C for 2 min. The total running time for each sample was 13 min.

2.2.2. Thermal properties

The thermal properties of all samples were determined by a differential scanning calorimeter (DSC Star® System; Mettler Toledo AG, Switzerland). Approximately 12 mg of flour was put directly into an aluminum pan using a flour to water ratio of 1:3 (w/w). The pan was sealed and equilibrated 1 h at room temperature before the analysis. The DSC scanning temperature range was set at 25-95°C using a heating rate of 10°C/min. The DSC was calibrated using indium as a standard and an empty aluminium pan as reference (Cham & Suwannaporn, 2010). The parameters were analyzed using STARe evaluation software v12.10 (Mettler Toledo AG, Switzerland).

2.3. *In vitro* digestibility of hydrolyzed-HMT rice flour

2.3.1 Rapid digestible starch (RDS) and slow digestible starch (SDS) content

An enzyme solution was freshly prepared by adding porcine pancreatic α-amylase (Sigma A-3176, Sigma-Aldrich, UK; 16 U/mg) (1.5 g) in a sodium acetate buffer (pH 5.2) (10 mL). The mixture was incubated at 37°C for 10 min and centrifuged (1500 g) for 10 min. The supernatant was transferred into a beaker and mixed with amylo-glucosidase (Sigma A-7095, Sigma-Aldrich, UK; 300 U/mL) at 8:1 (v/v) (Mutungi et al., 2011). The modified rice flour (100 mg) was suspended in a 0.1 M sodium acetate buffer (21 mL) and incubated at 37°C with continuous shaking (200 strokes/min) for 15 min. The freshly prepared enzyme solution (1.6 mL) was added to the suspension, mixed for 1 min and incubated at 37 °C in a shaking water bath (200 strokes/min). After incubation for 20 and 120 min, an aliquot (0.2 mL) was taken and added into absolute ethanol (4 mL), mixed well and centrifuged (5000 g) for 10 min. The supernatant was then collected for RDS and SDS determination. The glucose content was measured by adding a glucose oxidase-peroxidase assay kit (GOPOD, Megazyme International,

Ireland) into the aliquot and incubated at 50°C for 20 and 120 min. The aliquot was then measured in a spectrophotometer at 510 nm absorbance. Starch fractions that were digested (measured as % glucose) within 20 min and 20–120 min were calculated as RDS and SDS (Lin et al., 2009; Englyst et al., 1992).

2.3.2 Resistant starch (RS) content

A flour sample (100 mg) was weighed into a centrifugal tube and a KCl-HCl buffer (pH 1.5) (10 mL) was added. Pepsin solution (0.2 mL) was added into the mixture in order to remove protein. The pepsin solution was prepared by adding pepsin (P-7125, Sigma-Aldrich, UK) (1 g) in the KCl-HCl buffer (10 mL). The solution was mixed well and incubated in a shaking water bath (40°C) for 60 min and cooled down to room temperature. The solution was then added with a 0.1 M Trismaleate buffer (pH 6.9) (9 mL) and mixed with a pancreatic α-amylase solution (1mL). The pancreatic α-amylase solution was prepared by adding pancreatic α-amylase (A-3176, Sigma-Aldrich, UK) (40 mg) into a tris-maleate buffer (1 mL). The solution was incubated at 37°C for 16 h. The digested sample was centrifuged and the sediment was washed with distilled water (10 mL) and centrifuged. After that, the precipitate was mixed with distilled water (3 mL) and 4 M KOH (pH 4.75) (3 mL) for 30 min at room temperature. Then, 2 M HCl (5.5 mL) and 0.4 M sodium acetate buffer (pH 4.75) (3 mL) were added. Next, amyloglucosidase (A-7095, Sigma-Aldrich, UK) (80 µL) was incubated (60°C) for 45 min and centrifuged, and the supernatant was collected. The residue was washed with distilled water and centrifuged, and the supernatant was combined to make 100 mL. Sample solutions (0.1 mL) were pipetted into a test tube and added with glucose oxidase-peroxidase kit reagent (GOPOD, Megazyme International, Ireland) (3 mL), mixed well, and incubated (50°C) for 20 min. The solution was measured by a spectrophotometer using absorbance 510 nm against a blank reagent. The resistant starch content

was calculated as mg of glucose x 0.9. The standard curve was determined using known glucose concentrations (Goni et al., 1996).

2.4. Antioxidative activity of hydrolyzed-HMT rice flour

A flour sample (1 g) was extracted using an extraction solvent (methanol:water, 80:20 v/v) (25 mL), mixed for 24 h and centrifuged (2500 g) for 10 min. The collected supernatant was analyzed for antioxidant activity using the following assays:

2.4.1. ABTS radical cation decolorization assay

For scavenging activity determination, a 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid radical (ABTS) (Sigma-Aldrich, UK) solution was prepared by reacting a 7 mM aqueous solution of ABTS with potassium persulfate in the dark at room temperature for 16 h before use. The ABTS solution (1.5 mL) was added into the extracted sample (1.5 mL), mixed well and measured at 734 nm absorbance using a spectrophotometer. The result was expressed as Trolox equivalent antioxidant capacity (TEAC) in μM of Trolox/g flour (Jeng et al., 2012).

2.4.2. DPPH radical scavenging assay

The scavenging effect on the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical was measured according to Brand-Williams et al. (1995). The working solution of 0.2 mM DPPH was prepared by dissolving DPPH (3.94 mg) in methanol (50 mL). The extracted sample (1.5 mL) was added to the DPPH working solution (1.5 mL). The mixture was left at room temperature for 30 min and was measured at 517 nm absorbance using a spectrophotometer. The scavenging effect was expressed as Trolox equivalent antioxidant capacity in μM of Trolox/g flour.

2.5.

231 **2.5. *In vitro* batch culture fermentation**

232 *In vitro* fermentation describes the method used by Costabile et al. (2015). Experiments
233 were carried out using fresh faecal samples from three healthy donors (1 female aged 26 and 2
234 males aged 25 and 31). Donors never had any metabolic or gastrointestinal diseases, did not take
235 any probiotic/prebiotic supplements or antibiotics within the last 6 months. Each donor provided
236 written consent and filled in a standard questionnaire regarding their health status, drug use,
237 clinical anamnesis and lifestyles. This study was approved by the University of Reading
238 Research Ethics Committee (UREC 15/20). Each faecal sample was diluted to 1:10 (w/w) with
239 0.1 mol/L of phosphate buffer solution (PBS) at pH 7.4 and then homogenized (240 paddle
240 beats/min) (Stomacher 400, Seward, West Sussex, UK) for 2 min. The solution was placed in an
241 anaerobic jar (AnaeroJarTM 2.5 L, Oxoid Ltd) equipped with a gas generating kit
242 (AnaeroGenTM, Oxoid). A flour sample (0.5 g) was added into an anaerobic fermenter within 15
243 min of voiding (Rycroft et al. 2001). The experiment was run in triplicate (3 vessels per donor).
244 Oligofructose-enriched inulin (Orafti®Synergy1, Belgium) (0.5 g) was used as a positive control
245 and a vessel without any sample was used as a negative control. The fermentation system was
246 run for 24 h. The sample solution was collected at 0, 4, 8, and 24 h to analyse for bacterial
247 populations and short-chain fatty acids.

248 2.5.2. Short-chain fatty acids (SCFAs) analysis

249 A cell-free supernatant was obtained by centrifuging (13,000g) a sample solution
250 (obtained from section 2.5.1) (1 mL) for 10 min and filtering it through a 0.22 μm membrane
251 (Millipore, Cork, Ireland) directly into a vial. The SCFA content was measured by an ion
252 exclusion high performance liquid chromatograph (LaChrom, Merck Hitachi, Dorset, UK)
253 equipped with a pump (L-7100, Merck Hitachi, UK), RI detector (L-7490, Merck Hitachi, UK)

with a wavelength of 210 nm and an auto-sampler (L-7200, Merck Hitachi, UK). The sample solution (20 µL) was taken and injected into the HPLC with a prepacked Rezex ROA-organic acid H+ 80% (300 x 7.8 mm) column (Phenomenex Inc, UK), at a flow rate of 0.5 mL/min at 84ºC. H_2SO_4 (2.5 mM) was used as eluent. The amount of SCFAs (acetic, propionic and butyric) was calculated using standard samples at concentrations of 12.5, 25.0, 50.0, 75.0 and 100.0 mM. 2-ethylbutyric acid (20 mM) was used as an internal standard. All chemicals were provided by Sigma-Aldrich (Dorset, UK).

2.5.3 Enumeration of faecal bacteria population by fluorescence *in situ* hybridization (FISH)

FISH was analyzed following the method of Costabile et al. (2015). The oligonucleotide probe (Sigma-Aldrich, UK) was designed to target specific regions of 16S rRNA. The probe was commercially synthesized and labeled with fluorescent dye Cy3 (Sigma-Aldrich, UK). The probes used in this study are detailed in Table 1. The bacterial groups to be enumerated were (1) Eub338 I–II–III for the total number of bacteria, (2) Bif164 for *Bifidobacterium spp*., (3) Lab158 for *Lactobacillus-Enterococcus spp*., (4) Bac303 for the *Bacteroides-Prevotella* group and (5) Chis150 for the *Clostridium histolyticum* subgroup.

Table 1

2.6 Preparation of potential synbiotic microencapsulation by spray drying

The stock cell solution of *L. plantarum* TISTR 1465 (Thailand Institute of Scientific and Technological Research, Thailand) was freshly prepared. A pure lyophilized culture was thawed, suspended in 0.85% sterile saline (2 mL) and incubated at 37°C for 24 h. When the liquid was turbid, *L. plantarum* (1 mL) was taken, activated in MRS broth (9 mL) and incubated at 37°C for 24 h. The culture was collected by centrifugation (6000 g) for 10 min in the early stationary

277 phase. The bacterial pellets were washed and centrifuged 3 times with sterile saline (10 mL). The
278 final precipitate was suspended in sterile saline (5 mL) (Zhao et al., 2008). The cell concentration
279 used was approximately 10^{10} CFU/mL.

280 Native and all hydrolyzed-HMT rice flours (hydrolyzed for 8, 24, 36 h then HMT) were
281 used as wall materials for *L. plantarum* encapsulation. Gum arabic and maltodextrin were used
282 as control carriers. The carrier solution was prepared by mixing wall material in water to obtain a
283 final concentration of 20% (w/w). The mixture was then homogenized using a two-stage high
284 pressure homogenizer (5000 psi) (15MR-8TA, APV Gaulin, Inc., MA, USA). Previously
285 prepared stock cell solution (approximately 10^{10} CFU/mL) (5 mL) was mixed with Tween 80
286 (0.2 mL), followed by the carrier solution (94.8 mL). The mixture was stirred at 20°C for 20 min
287 and immediately fed into a spray dryer (GEA Niro, A/S, DK-2860, Soeborg, Denmark). The
288 inlet temperature (140±5°C) and outlet temperature (70±5°C) were obtained from a previous
289 experiment with the highest survival rate (data not shown). The spray-dried powder was
290 collected from the base of the cyclone, put in a sealed plastic bag and stored at 4ºC for the next
291 study.

292

293 **2.7. Enumeration of *L. plantarum* in the microcapsules**

294 2.7.1. Viability of *L. plantarum* after spray drying and storage

295 The viability of *L. plantarum* in the microcapsules was determined immediately after
296 spray drying and after storage at 4ºC for 30 and 90 days. Spray-dried powder (1 g) was
297 suspended in 0.1% peptone water (w/w) (9 mL) and homogenized by vortex for 10 min at room
298 temperature to ensure complete dissolution of the powder. A sample solution (1 mL) was plated
299 on MRS agar and incubated anaerobically at 37°C for 48 h (Yonekura et al., 2014).

300 2.7.2. Viability of *L. plantarum* under *in vitro* gastrointestinal condition

301 Simulated gastric fluid (SGF) was prepared by adding porcine pepsin (P-7125, Sigma-
302 Aldrich, UK) (3 g) in salt solution (1 L). Salt solution was prepared by adding 125 mM NaCl
303 (7.305 g), 7 mM KCl (0.52 g) and 45 mM $NaHCO_3$ (3.78 g). Distilled water was added to make
304 a volume of 1 L and its pH adjusted to 2.5 with 0.1N HCl. Synbiotic powder (1 g) was added
305 into test tube that contained pre-warmed (37°C), freshly filtered, sterilized SGF (9 mL) and
306 mixed well. The aliquot was incubated in a water bath (37°C) for 90 min. Bacterial cells were
307 collected and washed with 0.85% NaCl by centrifugation (6000 g) for 10 min. After that, the cell
308 pellets were resuspended in simulated intestinal fluid (SIF). SIF was prepared by adding
309 pancreatin (A-3176, Sigma-Aldrich, UK) (1 g) and bile extract (B-8631, Sigma-Aldrich, UK)
310 (1.5 g) in salt solution (1L) and its pH adjusted to 6.5 using 1 N NaOH. The aliquot was mixed
311 and incubated at 37°C. The digested aliquot (1 mL) was collected at 0, 90, 120 and 180 min and
312 added into normal saline (9 mL). The solution (1 mL) was then plated on MRS agar and
313 incubated at 37°C under anaerobic conditions for 48 h (Grimoud et al., 2010).

314

315 **2.8. Scanning electron microscopy**

316 The morphology of the spray-dried microcapsules was determined by scanning electron
317 microscopy (JM-560LV model, JEOL, Japan). Briefly, the encapsulated *L. plantarum* was fixed
318 to the sample slide with 2.5% glutaraldehyde for 2 h, and washed with 0.1 M phosphate buffer
319 (pH 7.2). The sample was fixed again with 2% tetroxide for 2 h, washed with deionized water
320 and dehydrated by increasing the concentrations of ethanol solutions (50%, 70%, 80%, 90% and
321 100%). The dried powder was spread thinly onto a double-sided carbon adhesive disc and then

anchored to the electron microscopy stub. The specimen was then coated with gold and examined under scanning electron microscope.

2.9. Data analysis

Data was analyzed statistically using SPSS Version 16. The experimental data of hydrolyzed-HMT rice flour properties and enumeration of microencapsulated *L. plantarum* were analyzed by ANOVA and Duncan's multiple rank tests. The differences between the bacterial counts, substrates and SCFAs profiles at 0, 4, 8 and 24 h of fermentation were tested using ANOVA with Tukey's post-test (P <0.05). All analysis were performed using a GraphPad Prism 5.0 (GraphPad Software, LaJolla, CA, USA).

3. Results and discussion

3.1. Pasting and thermal properties of hydrolyzed-HMT rice flour

Black waxy and red jasmine rice flour (6.5 and 18.7% amylose content, respectively) were modified using a debranching enzyme followed by heat-moisture treatment (HMT). A decrease in pasting parameters was identified by the increase in starch crystallinity that limited starch swelling (Figure 1a, 1b). Hydrolysis treatment is reported to promote an increase of low molecular weight starch that easily formed a crystalline structure after HMT (Polesi & Sarmento, 2011). Higher paste viscosity increased noticeably in the hydrolyzed-HMT of red jasmine rice flour (higher amylose content). Breakdown was observed to be substantially decreased, which indicated more rigidity and resistance to shearing of the modified granules. The thermal properties of gelatinization are used as thermal stability indices of starch granules. A higher gelatinization endotherm indicates more rigidity, more crystallinity and less swelling of starch granules. All hydrolyzed-HMT rice flour had higher thermal properties than the native flour

($p<0.05$) (Figure 1c, 1d). Pullulanase, the debranching enzyme, attacked the 1, 6 glucosidic linkages of amylopectin, generating a more linear chain (Reddy et al., 2015; Wong et al., 2007). Later, HMT rearranged these linear chains into a more perfect crystal.

Figure 1

3.2. *In vitro* digestibility of hydrolyzed-HMT rice flour

The enzyme digestibility of hydrolyzed-HMT rice flour was monitored by time needed to convert starch to glucose in a simulated gastrointestinal environment. A significant increase in SDS and RS and a decrease in RDS were noticeable in the hydrolyzed-HMT of black waxy rice flour, while a much lower effect was found in the non-waxy type (Table 2). Waxy rice was more susceptible to pullulanase, accounting for its higher α-1,6 branch point. Hydrolyzed 36 h-HMT black waxy rice flour showed a desirable quality as it could produce high SDS and RS and low RDS. SDS and RS that can pass through the stomach into the small and large intestines are desirable, playing an important role in both prebiotic and protection wall materials in this study.

Table 2

In this study, pullulanase was used to hydrolyze ungelatinized swollen starch granules without liquefaction. Hydrolysis occurred mostly on a solid surface (Jung et al., 2013) that is not strong enough to induce the formation of RS. RS required linear polymers of a minimum chain length of approximately 10 DP to form double helices (Mutungi et al., 2010; Miao, Jiang, & Zhang, 2009). Chain mobility improved during hydrothermal process that enabled crystalline rearrangement, hence increasing indigestible starch formation. Limited enzyme reaction caused SDS to increase and then become stagnant at 24-36 h because no liquefaction occurred. The

result agreed well with Wong et al. (2007) in that linear long-chain dextrin did not increase much in non-gelatinized sago starch treated with pullulanse for 24 h as compared to gelatinized starch.

3.3. Antioxidant properties

The DPPH and ABTS scavenging activity of all samples decreased drastically after modification (Figure 2). Soaking during hydrolysis incubation and spray drying deteriorated some water-soluble pigments of colored rice flour. Modified black waxy rice flour drastically decreased antioxidant activity in comparison with red jasmine rice flour. However, a higher level of antioxidant activity in black waxy rice flour was still retained, accounting for its high initial content in both free and bound forms. Protocatechuic acid, a phenolic compound in bound form, was only found in black rice, whereas ferulic, *p*-coumaric and vanillic acid were found in both red and black rice varieties (Sompong et al., 2011).

Figure 2

3.4. Viability of *L. plantarum* after spray drying and storage

After spray drying, the viability of *L. plantarum* in all spray-dried samples was not much different (9.36-9.71 log CFU/g) (Table 3). Yonekura et al. (2014) was also reported non-significantly different in *L. acidophilus* survival after spray drying with sodium alginate, hydroxypropylmethyl cellulose and chitosan (8.90-8.99 log CFU/g). However, a noticeable difference in survival rates was observed in longer storage times (90 days). Maltodextrin showed the lowest survival rate (44.68%) while hydrolyzed 36h-HMT showed the highest survival rate (89.56%). The modified black waxy rice flour protected *L. plantarum* better than red jasmine. Protection often correlated with high glass transition temperature of wall materials which provide

391 stability for bacteria enclosed within a glassy matrix (Crowe et al., 1998; Leslie et al., 1995). The
392 increase in glass transition temperatures can provide stabilization via the fixation of bacterial
393 cells in a glassy state during spray drying (Perdana et al., 2014). Encapsulated microcapsules
394 from hydrolyzed-HMT rice flour showed good protection properties supported by high bacterial
395 survival even after a long storage time (90 days, 4ºC). Hydrolyzed 36h-HMT of black waxy rice
396 flour was selected to use as wall material throughout later experiments.

397 **Table 3**

398

399 **3.5. Survival of *L. plantarum* in *in vitro* gastrointestinal environment**

400 The sharp reduction in the viable cell count was observed in the stage of SGF (90 min).
401 At this stage, the survival rate of *L. plantarum* in encapsulated powder with hydrolyzed 36h-
402 HMT, gum arabic and no carrier (free cell) were 88.02% (8.07 Log CFU/g), 75.38% (6.12 Log
403 CFU/g) and 36.86% (3.34 Log CFU/g), respectively (Figure 3). At the end of SIF (180 min), the
404 hydrolyzed 36h-HMT showed much higher survival and was stabilized throughout the digestion
405 process. The survival rate of hydrolyzed 36h-HMT, gum arabic and no carrier (free cell) were
406 81.56% (7.48 Log CFU/g), 58.1% (4.72 Log CFU/g) and 0%, respectively. Without any carrier,
407 no viable cells of *L. plantarum* were found, as they are sensitive to the acid-bile condition. Gum
408 arabic, a frequently used commercial carrier, is a soluble fiber consisting mostly of the carboxyl
409 group. Spray-dried particles with gum arabic dissolved more easily in SGF and SIF solutions,
410 hence lowering its prebiotic property and probiotic protection. Xing et al. (2015) also suggested a
411 complex wall material that consisted of porous starch (10%), mannitol (3%) and glycerol (2%) to
412 protect *L. acidophilus* against the intestinal system and refrigerated storage. The microparticles
413 made from hydrolyzed 36h-HMT showed SDS and RS properties as some particles remained

414 after SGF and SIF conditions. These particles protect probiotic cells survival and help get these
415 cells through the lower part of the gastrointestinal tract to produce SCFAs.

416 **Figure 3**

417 **3.6. Scanning electron microscopy (SEM) of microencapsulated aggregates**

418 Starch granules of the hydrolyzed 36h-HMT rice flour remained ungelatinized, which
419 were observed as polyhedral shapes formed in spherical aggregates. The spherical aggregates
420 were produced when starch granules of small size were rapidly dehydrated by spray drying with
421 low amounts of bonding agents (Zhao & Whistler, 1994) (Figure 4). The protein in the flour was
422 reported to promote the formation of these aggregates (Avila-Reyes et al., 2014). *L. plantarum*
423 was scattered within the interstitial spaces and at the periphery of the aggregates (Figure 4).
424 These aggregates carried the microorganisms and reduced their injury and mortality. The
425 aggregates of microcapsules from hydrolyzed 36 h-HMT were similar to those encapsulated with
426 various carbohydrates or colloids, such as inulin, gum arabic, maltodextrin and native starch
427 (Avila-Reyes et al., 2014; Rodríguez-Huezo et al., 2007; Desmond et al., 2002)

428 **Figure 4**

429

430 **3.7. Short chain fatty acids (SCFAs)**

431 SCFAs were generated by the metabolism of faecal bacteria using wall materials as
432 substrates. SCFAs were analyzed in comparison to a commercial prebiotic as a positive control
433 (Table 4). Indigestible carbohydrates were consumed and converted into SCFAs by intestinal
434 microbes (Casterline et al., 1997). SCFAs have an important role in maintaining gut health and
435 host energy metabolism (den Besten et al., 2013; Dongowski et al., 2005). SCFAs of the
436 hydrolyzed 36 h-HMT and the positive control were much higher than the negative control at

every fermentation time ($p<0.01$). Hydrolyzed 36 h-HMT was able to ferment at a higher rate and produced higher amounts of SCFAs than the positive control. High amounts of acetate were detected and rapidly increased after 4 h of fermentation. Acetate was reported as a main metabolized product of starch and arabino-oligosaccharides by faecal bacteria (Vigsnaes et al., 2011). Acetic acid was the main metabolite produced by bifidobacteria in prebiotic stimulation with oligofructose (Van der Meulen et al., 2006). Propionate and butyrate showed gradual increases during faecal fermentation. Butyrate was formed by free acetate as a precursor of the conversion by butyryl CoA from colon bacteria (Scott, Duncan, & Flint, 2008).

Table 4

3.8. Modulation of bacterial populations by FISH

In comparison with the Orafti®Synergy1 (a commercial prebiotic, inulin based), fermentaion of hydrolyzed 36 h-HMT sample caused significantly higher numbers of *Lactobacilli* (enumerated by Lab158 probe) at 8 h (from Log 7.91±0.02 to 7.50±0.05, $P < 0.05$) and 24 h (from Log 8.40±0.50 to 7.47±0.08, $P < 0.01$) (Figure 5). However, there was no significant difference between hydrolyzed-HMT and Orafti®Synergy1 in the other bacteria enumerations.

In comparison with the negative control, the fermentation of hydrolyzed 36h-HMT showed significantly higher numbers of lactobacilli (Lab158), *Bifidobacterium* ssp. (Bif164) and total bacteria (Eub338 I-II-III) (Figure 5). Numbers of lactobacilli in hydrolyzed 36 h-HMT fermentation was higher than the negative control for every fermentation time; 4 h (from Log 7.85±0.07 to 7.30±0.01, $P < 0.05$), 8 h (from Log 7.91±0.02 to 7.03±0.11, $P < 0.01$) and 24 h (from Log 8.40±0.50 to 7.03±0.20, $P < 0.05$). A significant increase in the Bif164 group was

460 also found in hydrolyzed 36 h-HMT in comparison to the negative control after being fermented
461 for 8 h (from Log 9.14±0.51 to 8.58±0.20, $P < 0.05$) and 24 h (from Log 9.50±0.11 to 8.10±0.20,
462 $P < 0.01$). The total bacteria enumerated with Eub338 I-II-III showed significant increases at 8 h
463 (from Log 9.43±0.40 to 8.63±0.21, $P < 0.01$) and 24 h (from Log 9.60±0.28 to 8.05±0.06, $P <$
464 0.001). Only the numbers of the Chis150 group decreased at 24 h (from Log 7.22±0.20 to
465 6.65±0.31, $P < 0.01$). No significant changes in *Bacteroides-Prevotella* numbers (Bac303) were
466 found for any fermentation time point or substrate.

467 The results indicate the prebiotic properties of hydrolyzed 36h-HMT that aid the growth
468 of beneficial probiotics (mainly *Lactobacillus* spp. and *Bifidobacterium* spp.) after fermentation,
469 as quickly as 8 h earlier than the negative control. Moreover, a better or comparable prebiotic
470 quality of hydrolyzed 36 h-HMT was also found in comparison with commercial prebiotic of
471 inulin base. This result corresponds well with the SCFAs production (Table 4). The
472 polysaccharide from wheat dextrin was also reported to enhance numbers of Lactobacillus in *in*
473 *vitro* batch fermentation (Noack et al., 2013). A significant increase of *Lactobacillus* in the mice
474 faeces was also found after applying amylomaize starch in the rat diet (Wang et al., 2002).

475 **Figure 5**

476

477 **4. Conclusion**

478 Encapsulated formulation with slow digestible colored rice flour could be used as a
479 cheaper alternative functional food ingredient. Hydrolyzed-HMT of colored rice flour obtained
480 both potential prebiotic and synbiotic properties due the antioxidative property. Moreover, its
481 activity was stable throughout the 90-day studied shelf life further studies of these novel
482 formulations are needed to determine in vivo cell delivery performance and efficacy.

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